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REMARKS

Claims 15-19 and 37-61 are pending. Claims 42-56 are under examination. Claims 42 and 47 have been amended. support for the amendments can be found throughout the specification and the claims as filed. In particular, support for the amendments can be found in original claim 1 and on page 8, lines 29-31; page 11, lines 6-13; page 13, line 32, to page 14, line 2; page 15, lines 1-13; and page 31, lines 23-33. Accordingly, these amendments do not raise an issue of new matter and entry thereof is respectfully requested.

Regarding the Restriction of Claims

Applicant understands that the previous restriction of the claims has been maintained. However, Applicant would like to make observations about some of the comments on maintaining the restriction. First, it is asserted that the originally claimed "common ligand to a conserved site in a receptor family" would read on a common ligand that is a cofactor or mimic thereof and/or a common ligand that binds in the cofactor binding site and would not necessarily include common ligands "wherein said common ligand competes for cofactor binding." However, it is unclear how a common ligand that binds in the cofactor binding site would not encompass a common ligand that competes for cofactor binding since a common ligand and cofactor that bind to the same site would by definition exhibit competitive binding.

Secondly, it is asserted that the addition of the limitation "wherein said common ligand competes for cofactor binding" adds a process limitation to the claimed invention that was not previously there. Applicant maintains that the phrase "wherein said common ligand competes for cofactor binding" is a functional characteristic of the common ligand and not a process step of the claimed method. With regard to the assertion that the addition of this phrase adds a limitation that was not previously there, it is unclear to Applicant why inclusion of a limitation would not be permitted since a limitation would be considered to limit the scope of the previous claim, albeit, as discussed above, there would be significant overlap between those common ligands that bind in the cofactor binding site and those that compete for cofactor binding. Absent the ability to amend claims to incorporate limitations over previously pending claims, it is unclear how Applicant can amend the claims to further prosecution.

Thirdly, it is asserted that "[O]ne could easily envision art that would read on a common ligand that is a cofactor or mimic thereof and/or a common ligand that binds in the cofactor binding site that would ***not*** read on a common ligand 'wherein said common ligand competes for cofactor binding.'" Applicant respectfully disagrees that one can easily envision a common ligand that is a cofactor, or a mimic thereof, that would not compete for cofactor binding. Such a cofactor or mimic thereof would compete

for cofactor binding since the cofactors bind to the same cofactor binding site. Furthermore, a common ligand that binds in the cofactor binding site would be expected, with rare exception, to similarly compete for cofactor binding. Thus, Applicant maintains the position of record that examination of these claims would not be an undue burden.

Rejections Under 35 U.S.C. § 112, First Paragraph

The rejection of claims 42-56 under 35 U.S.C. § 112, first paragraph, as allegedly lacking sufficient written description is respectfully traversed. Applicant respectfully maintains that the specification provides sufficient description and guidance to convey to one skilled in the art that Applicant was in possession of the claimed invention at the time the application was filed.

With regard to terms recited in the claims such as "cofactor binding site," "cofactor or mimic thereof," "second ligand," "third ligand," "substrate binding site," "enzyme family," and "linker," the Office Action asserts that the terms are defined or discussed in the specification but the definitions "are very broad and open-ended." The issue for meeting the written description requirement is not whether the terms are considered to be broad but whether one skilled in the art, based on the teachings in the specification and what was well known to those skilled in the art, would have understood that Applicant was in possession of the claimed invention at the time the application was filed. Applicant maintains that

the terms recited in the claims are well known to those skilled in the art or defined in the specification, as acknowledged in the Office Action, and therefore satisfy the written description requirement.

Although Applicant maintains that the specification provides sufficient written description for the previously pending claims, claims 42 and 47 have nevertheless been amended to explicitly incorporate language reciting the identification of two bi-ligands, which are used to generate the claimed bi-target ligand. As taught in the specification, a bi-target ligand is identified by identifying a first bi-ligand and a second bi-ligand and linking the first and second bi-ligand to form a bi-target ligand (page 47, line 16, to page 48, line 16). Applicant points out that the language incorporated into the amended claims and explicitly reciting the identification of the bi-ligands has been acknowledged in related application serial No. 09/765,696 as having sufficient description and guidance in the specification to convey to one skilled in the art that Applicant was in possession of the claimed method of identifying bi-ligands at the time the application was filed. The specification further teaches methods of generating a bi-target ligand by linking two bi-ligands (page 47, line 16, to page 48, line 16).

With regard to the indication in the Office Action that no structure of the identified bi-target ligand, it is respectfully submitted that the claims are

directed to methods for identifying a bi-target ligand. Nevertheless, the claims do recite terms such as "common ligand or mimic thereof" that clearly provide a structural and functional activity of the bi-target ligands to be identified by the claimed method.

The Office Action refers to *University of California v. Eli Lilly & Co.* (119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997) and indicates that a "description of a compound in terms of its function fails to distinguish the compound from others having the same activity or function." Applicant respectfully points out that the claims are directed to methods of identifying compounds, not to compounds themselves.

Applicant maintains that the specification provides sufficient description and guidance to convey to one skilled in the art that Applicant was in possession the claimed methods for identifying a bi-target ligand at the time the application was filed. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

The rejection of claims 42-56 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement is respectfully traversed. Applicant respectfully maintains that the specification provides sufficient description and guidance to enable the claimed invention.

Although Applicant maintains that the specification provides sufficient description and guidance

to enable the previously pending claims, claims 42 and 47 have nevertheless been amended to explicitly incorporate language reciting the identification of two bi-ligands, which are used to generate the claimed bi-target ligand. As discussed above and taught in the specification, a bi-target ligand is identified by identifying a first bi-ligand and a second bi-ligand and linking the first and second bi-ligand to form a bi-target ligand (page 47, line 16, to page 48, line 16). Applicant points out that the language incorporated into the amended claims and explicitly reciting the identification of the bi-ligands has been acknowledged in related application serial No. 09/765,696 as having sufficient description and guidance in the specification to enable the claimed method for identifying a bi-ligand. The specification further teaches methods for generating a bi-target ligand by linking two bi-ligands (page 47, line 16, to page 48, line 16).

Applicant agrees with the assertion in the Office Action that compounds that interact with various enzyme targets were known in the art at the time of filing but respectfully disagrees with the assertion that only limited numbers of such compounds were known. Many ligands that bind to enzymes and enzyme cofactor binding sites were well known in the art at the time the application was filed, including enzyme inhibitors, many of which function as drugs for treating various diseases. As evidence that a number of ligands that bind to the cofactor binding site of enzymes were well known to those skilled in the art, attached herewith as Exhibit A is a reference by Radzicka

and Wolfenden, Methods Enzymol. 249:284-312 (1995), which describes enzyme inhibitors that bind to various enzyme targets.

With regard to the indication in the Office Action that no structure of the identified bi-target ligand is provided, it is respectfully submitted that the claims are directed to methods for identifying a bi-target ligand. Nevertheless, the claims do recite terms such as "common ligand or mimic thereof" that clearly provide a structural and functional activity of the bi-target ligands to be identified by the claimed method.

Applicant maintains that the specification provides sufficient description and guidance to enable the claimed methods for identifying a bi-target ligand. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

CONCLUSION

In light of the amendments and remarks herein, Applicant submits that the claims are now in condition for allowance and respectfully requests a notice to this

effect. The Examiner is invited to call the undersigned agent if there are any questions.

Respectfully submitted,

September 18, 2003
Date

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[11] Transition State and Multisubstrate Analog Inhibitors

By ANNA RADZICKA and RICHARD WOLFENDEN

Transition State Stabilization by Enzymes

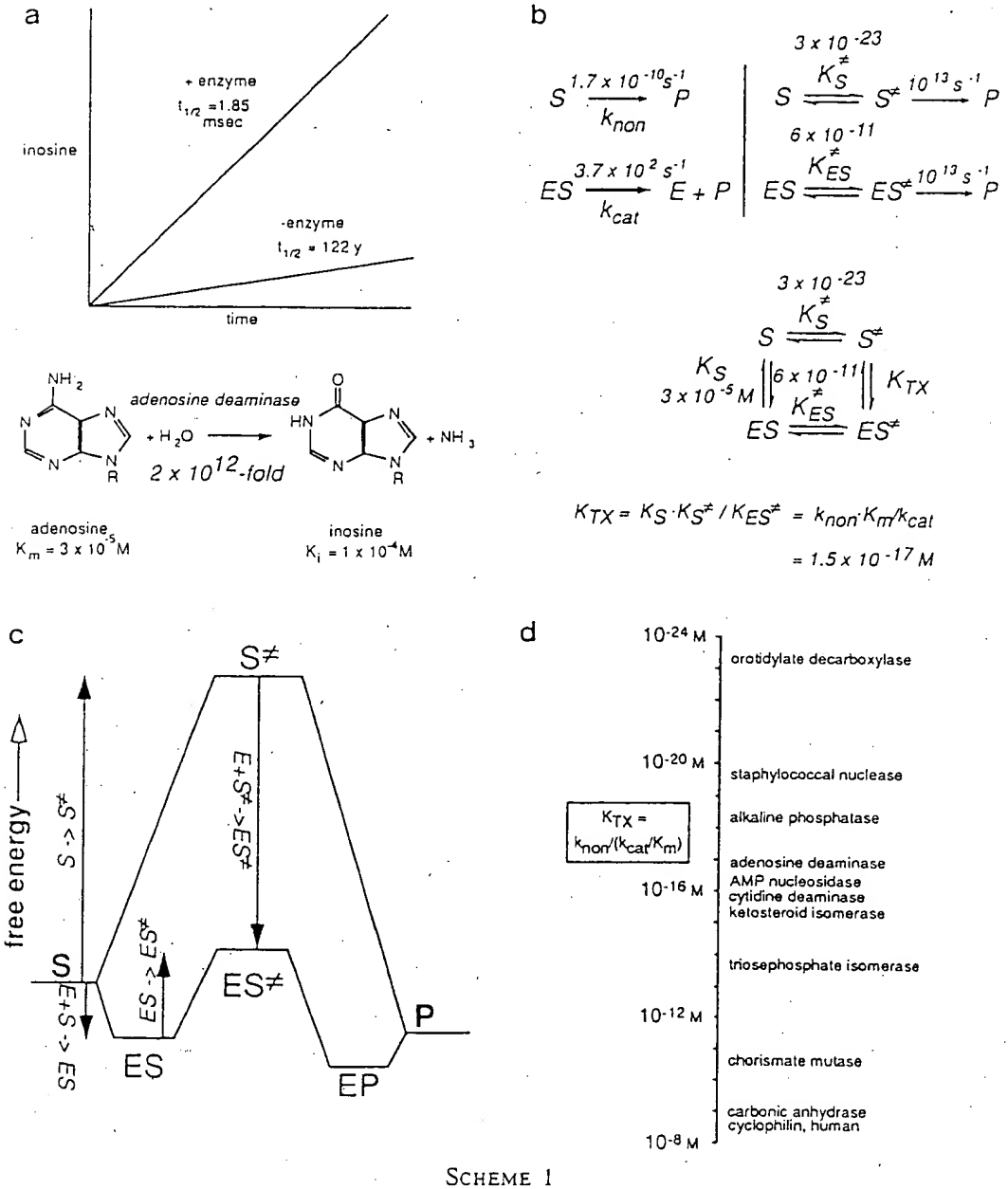
To lower the energy barrier that limits the rate of a reaction, a catalyst must bind the altered substrate in the transition state ($S^{\ddagger}$) more tightly than it binds the substrate in the ground state (S). In the moment, lasting perhaps 1 msec, during which the catalytic event occurs, binding is enhanced by a factor that equals or surpasses the factor by which the catalyst enhances the rate of the reaction.[1]

Scheme 1 illustrates this principle by comparing the rate of deamination of adenosine in the presence and absence of calf intestinal adenosine deaminase (Scheme 1a). The central postulate of transition state theory is that in the ground state the substrate S (in the nonenzymatic reaction), or the enzyme–substrate complex ES (in the enzyme-catalyzed reaction), exists in a state of equilibrium with a transition state ($S^{\ddagger}$ or $ES^{\ddagger}$, respectively) situated at the top of a potential energy barrier, from which its chances of going forward to products, or backward to substrates, are equal (Schemes 1b and 1c).

The rate of decomposition of any transition state to products is equal to kT/h, a universal rate constant composed of Planck's constant k, Boltzmann's constant h, and the absolute temperature T; and it has a value of approximately 0.62×10^{13} sec^{-1} at room temperature. At any given temperature, rates of reactions differ according to the difference between their equilibrium constants for reaching the transition state. That equilibrium constant is always unfavorable, even for the fastest enzyme reactions. Thus, for carbonic anhydrase, with a turnover number of roughly 10^6 sec^{-1},[2] the value of $K_{ES^{\ddagger}}$ is about 10^{-7}. For calf intestinal adenosine deaminase, a more conventional enzyme with a turnover number of 375 sec^{-1}, the value of $K_{ES^{\ddagger}}$ is approximately 6×10^{-11}. At neutral pH in water, adenosine is deaminated very slowly ($k_{non} = 1.8 \times 10^{-10}$ sec^{-1}; $t_{1/2} = 122$ years),[3] and the value of $K_{S^{\ddagger}}$ is approximately 3×10^{-23}. The ratio of $K_{ES^{\ddagger}}$ to $K_{S^{\ddagger}}$ matches the rate enhancement of 2×10^{12}-fold. Scheme 1b shows that K_S is related to K_{TX} by this same ratio, where K_S is the dissociation constant of the enzyme–substrate com-

[1] R. Wolfenden, *Nature (London)* **223**, 704 (1969).
[2] H. Steiner, B. H. Johnson, and S. Lindskog, *Eur. J. Biochem.* **59**, 253 (1975).
[3] L. Frick, J. P. Mac Neela, and R. Wolfenden, *Bioorg. Chem.* **15**, 100 (1987).

EXHIBIT A



SCHEME 1

plex and K_{TX} is the dissociation constant of the enzyme–substrate complex in the transition state. Evidently the substrate, initially bound with a dissociation constant of roughly 3×10^{-5} M, is bound with a dissociation constant of approximately 1.5×10^{-17} M in the transition state.

In the simple case described in Scheme 1, K_{TX} (expressed in moles/

liter) is equivalent to k_{non} (the first-order rate constant for the nonenzymatic reaction, usually expressed as sec^{-1}) divided by k_{cat}/K_m (the second-order rate constant for the enzyme reaction, usually expressed as $sec^{-1} M^{-1}$).

Second-order rate constants for enzyme reactions typically fall in the range between 10^5 and 10^7 $sec^{-1} M^{-1}$. Rate constants for the corresponding nonenzymatic reactions are distributed over a much wider range, with half-times that may be measured in tens of seconds or millions of years. Scheme 1d shows approximate values of K_{TX} for several enzymes, estimated from such measurements. The resulting affinities of $S^{\ddagger}$ are seen to be high. In most cases, the observed rate enhancement probably provides a conservative estimate of the levels of binding affinity that are achieved in the transition state. As has been shown elsewhere,[4] transition state affinity may be underestimated, from simple comparison of rates, if (1) the enzymatic and nonenzymatic reactions differ mechanistically in some fundamental respect, so that their transition states are unrelated in structure; or (2) the chemical mechanisms are the same, but the rate-limiting transition state is reached at a different point on the reaction coordinate in the enzymatic and nonenzymatic reactions. In both cases, simple comparison of rates results in underestimation of the ability of the enzyme to stabilize the transition state for the step that limits the rate of the nonenzymatic reaction.

This view of catalysis, focusing attention on a concrete structure rather than a process, implies that the catalytic power of enzymes lies in their extremely high affinity for unstable intermediates in substrate transformation, as opposed to the substrate in the ground state. The rate of the nonenzymatic reaction can be enhanced only if this difference in binding affinities exists. This principle also furnishes a practical basis for designing powerful enzyme antagonists, in the form of stable analogs of $S^{\ddagger}$. These inhibitors, usually termed "transition state analogs," can be designed to test alternative mechanisms by which the enzyme might act; that is, strong binding should be observed only for those inhibitors that resemble activated forms of the substrate that arise along the pathway of the enzyme reaction. Such inhibitors can be used to probe the source of the binding discrimination of the enzyme between the substrate in the ground state and that in the transition state, which lies at the heart of the catalytic process. Thus, exact structural observations on enzyme–inhibitor complexes should make it possible to identify the origins of the affinity of the enzyme for the inhibitor and, by inference, the interactions that stabilize

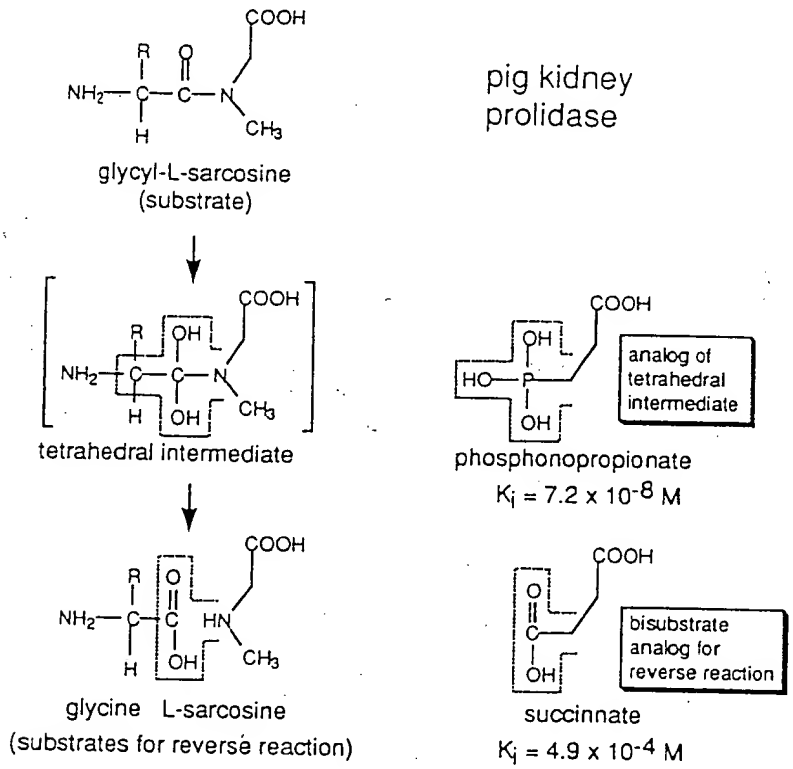
[4] For a review, see R. Wolfenden, *Annu. Rev. Biophys.* **5**, 271 (1976).

the actual transition state for the reaction, involving those amino acid residues of the enzyme that are directly involved in catalysis.

The transition state, by definition the highest point on the energy profile of a reaction, involves bond angles, bond distances, and electron distributions that can never be imitated precisely in any stable analog inhibitor. In addition, some compounds designed on this principle resemble reaction intermediates whose structures and energies may approach, but inevitably fall short of, that of the altered substrate in the transition state itself. For these reasons, the term "transition state analog" describes an ideal that will never be fully attained. Nevertheless, $S^{\ddagger}$ represents an ideal that is worth imitating, because a compound that shares even a few of the structural features that distinguish $S^{\ddagger}$ from S should be a very strong inhibitor, many orders of magnitude more strongly bound than the substrate or product. Potential transition state analog inhibitors have now been prepared against enzymes catalyzing reactions of every class (see Table I at the end of this chapter), and some show very high affinities. For example, 1,6-dihydroinosine (or nebularine 1,6-hydrate, described below) is bound by intestinal adenosine deaminase 3×10^8-fold more tightly than product inosine[5]; 3,4-dihydrouridine is bound by bacterial cytidine deaminase 2×10^9-fold more tightly than product uridine.[6]

A special kind of activation involves gathering of two or more substrates from dilute solution at the active site and their binding in an orientation appropriate for reaction. A multisubstrate analog inhibitor that incorporates binding determinants of two or more substrates within the same molecule may express a large entropic advantage in binding, as compared with the binding properties of analogs of the two substrates measured separately.[7] In principle[8] and in practice,[9] these effects can enhance reaction rates and inhibitor binding affinities by factors as large as 10^8 or more. Accordingly, given only that a compound is an exceptionally powerful inhibitor of a multisubstrate reaction, it may not be easy to decide whether its potency is due to some resemblance to the combined substrates, or to a chemically activated intermediate in which bonds are being made and broken. In some cases, such as that shown in Scheme 2, one inhibitor, tentatively identified as a multisubstrate analog inhibitor, is greatly sur-

[5] W. Jones, L. C. Kurz, and R. Wolfenden, *Biochemistry* **28**, 1242 (1989).
[6] L. Frick, C. Yang, V. E. Marquez, and R. Wolfenden, *Biochemistry* **28**, 9423 (1989).
[7] R. Wolfenden, *Acc. Chem. Res.* **5**, 10 (1972).
[8] M. I. Page and W. P. Jencks, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1678 (1971).
[9] W. M. Kati, S. A. Acheson, and R. Wolfenden, *Biochemistry* **31**, 7356 (1992).



SCHEME 2

passed in potency by another inhibitor containing a bond arrangement that resembles a hydrated intermediate in peptide cleavage.[10] The first of these compounds might be considered a multisubstrate analog, whereas the second has some of the structural features expected of a transition state.

Designing Transition State Analogs

Transition state and multisubstrate inhibitors have been prepared against enzymes catalyzing reactions of every class (see Table I). Each of the various devices by which enzymes are able to enhance the rates of reactions that they catalyze, including catalysis by approximation, general acid–base catalysis, catalysis by desolvation, nucleophilic catalysis, and catalysis by distortion, now appears to have been exploited in several inhibitors (for a review, see Wolfenden and Frick[11]). In what follows, we

[10] A. Radzicka and R. Wolfenden, *Biochemistry* **30**, 4160 (1991).
[11] R. Wolfenden and L. Frick, *in* "Enzyme Mechanisms" (M. I. Page and A. Williams, eds.), p. 9. Royal Society of Chemistry, London, 1987.

consider a few such inhibitors that have been grouped into several classes that emphasize their charge, reactivity with nucleophiles, hydrophobicity, or multisubstrate character.

Analogs of Anionic Intermediates

Isotope exchange studies have shown that many enzymes abstract protons from substrates, generating carbanionic or oxyanionic intermediates that undergo subsequent addition or rearrangement reactions. Inhibitory compounds resembling these species can be prepared by incorporation of stable oxyanionic substituents such as carboxylate groups.

Triose-phosphate isomerase, for example, was found to be strongly inhibited by 2-phosphoglycolate, analogous in structure to a suspected ene-diolate intermediate in the enzyme reaction. Judging from the effect of pH on K_i, the inhibitor appeared to be bound as a dianion.[12] However, ^{13}C and ^{31}P nuclear magnetic resonance experiments showed that the inhibitor was bound as the trianion, requiring that the enzyme have taken up a proton as binding occurred.[13] It was inferred that the EI complex resembled a species on the reaction pathway in which a basic group on the enzyme, Glu-165, had abstracted a proton from the substrate to form an ene-diolate intermediate, and that interpretation has been confirmed by X-ray crystallography of the EI complex.[14] The true affinity of the enzyme for the inhibitor is therefore several orders of magnitude higher than the K_i value had suggested. A comparable approach has been used to examine the complex formed between carboxypeptidase A and the multisubstrate analog inhibitor L-benzylsuccinate. From the dependence of K_i on pH, the inhibitor appeared to be bound as one of two possible monoanionic species involving the two carboxylic acid groups of the inhibitor.[15] When these groups were substituted with ^{13}C, resonances of the bound inhibitor showed that it was bound instead as the dianionic species, with concomitant release of a hydroxide ion from the protein.[16]

Barbituric acid ribonucleoside 5′-phosphate is an excellent inhibitor of orotidylate decarboxylase from yeast, presumably because one of its canonical forms resembles a zwitterionic intermediate formed during decarboxylation.[17] The resonance of ^{13}C at C-5, as well as the UV difference

[12] R. Wolfenden, *Biochemistry* **9**, 3404 (1970).
[13] I. D. Campbell, R. B. Jones, P. A. Keiner, E. Richards, S. G. Waley, and R. Wolfenden, *Biochem. Biophys. Res. Commun.* **83**, 347 (1978).
[14] E. Lolis and G. A. Petsko, *Biochemistry* **29**, 6619 (1990).
[15] L. D. Byers and R. Wolfenden, *Biochemistry* **12**, 2070 (1973).
[16] A. R. Palmer, P. D. Ellis, and R. Wolfenden, *Biochemistry* **21**, 5056 (1982).
[17] H. L. Levine, R. S. Brody, and F. H. Westheimer, *Biochemistry* **19**, 4993 (1980).

spectrum of the bound inhibitor, shows that it is bound in zwitterionic form, not as a covalent 5,6-adduct that would have been expected if the enzyme had acted by an alternative mechanism involving addition and elimination of an enzyme nucleophile.[18]

Analogs of Cationic Intermediates

Carbonium ion intermediates are generated during acid-catalyzed hydrolysis of glycosides, and also during the action of most glycosidases. In the enzyme reactions, a covalent glycosyl–enzyme intermediate may also be formed, but carbonium ions intervene during its formation and breakdown. Presumably for this reason, several glycosidases are strongly inhibited by 1-amino sugars whose protonated forms may form ion pairs with carboxylate groups at the active site that normally serve as a source of protons to the leaving group.[17]

Sterol methyltransferases are believed to involve nucleophilic attack by the sterol on the methyl function of *S*-adenosylmethionine. The methylated intermediate contains a positive charge on an adjacent tertiary carbon atom, and when this atom is replaced by nitrogen potent inhibition results.[2] Squalene synthetase is believed to generate a carbonium ion adjacent to a cyclopropane ring, by elimination of a pyrophosphoryl group. Rearrangement of the intermediate, followed by hydride transfer, leads to squalene. An analogous azasterol serves as a strong inhibitor.[12]

Electrophilic Analogs

Many hydrolases and transferases act by a double displacement mechanism, in which an enzyme nucleophile displaces part of the substrate to form a covalently bound intermediate that undergoes hydrolysis or transfer in a second step. Enzymes involved in carboxyl (or phosphoryl) transfer reactions are often susceptible to inhibition by analogs that undergo the first stage of reaction, typically forming a tetrahedral (or trigonal bipyramidal) intermediate but stop at that stage because they lack an appropriate leaving group. Thus, aldehyde analogs of peptides and amides, in which a hydrogen atom replaces the normal leaving group, are powerful inhibitors of proteases with cysteine[19] or serine[20] nucleophiles at the active site, forming hemiacetals whose stability reflects (1) the ability of the enzyme to stabilize tetrahedral intermediates in substitution and (2) the unusually favorable equilibrium constant for addition of nucleophiles to aldehydes.

[18] S. A. Acheson, J. B. Bell, M. E. Jones, and R. Wolfenden, *Biochemistry* **29**, 3198 (1990).
[19] J. O. Westerik and R. Wolfenden, *J. Biol. Chem.* **247**, 8195 (1972).
[20] R. C. Thompson, *J. Biol. Chem.* **12**, 47 (1973).

When such an enzyme is also specific for the leaving group, an additional advantage can be gained by using a ketone in which one substituent represents the acyl group and the other substituent represents the leaving group. Equilibria of addition of nucleophiles to ketones are much less favorable than for addition to aldehydes, but this effect can be offset by incorporating fluoro groups to promote electrophilic character. In this way, inhibitors of remarkable potency were developed for acetylcholinesterase and several proteases.[21]

Instead of mediating a double displacement reaction, some hydrolases catalyze direct transfer to the acceptor water. Aldehydes and ketones can also inhibit reactions of these kinds, not by addition of an enzyme nucleophile, but by addition of water itself to form a *gem*-diol that resembles a tetrahedral intermediate in direct water attack on the peptide bond. Demonstrated first for pepsin inhibitors containing the unnatural amino acid statone,[22] this mode of binding has also been observed in complexes of fluoroketones with carboxypeptidase A.[23]

Enzymes of the latter type are extremely strongly inhibited by a special class of compounds in which a $-P(=O)O^-—NH_2-$ group replaces the peptide bond, allowing four substituents (including an oxygen anion) to interact with the active site in a manner resembling an oxyanionic intermediate in peptide hydrolysis,[24,25] and this mode of binding has been confirmed by X-ray crystallography.[26] This combination of features confers on these compounds very high binding affinities that may have been surpassed only by the complex formed between methionine sulfoximine phosphate and glutamine synthetase (glutamate–ammonia ligase).[27]

Analogs of Nonpolar Intermediates

Ethanol enhances the rate of decarboxylation of pyruvate by a factor of 10^4–10^5, approaching the value observed for pyruvate dehydrogenase catalyzing the same reaction.[28] This increase in the nonenzymatic rate was ascribed to the greater stability of the neutral resonance of the reactive ylide intermediate in the organic solvent, as compared with the charged

[21] M. H. Gelb, J. P. Svaren, and R. H. Abeles, *Biochemistry* **24**, 1813 (1985).
[22] D. H. Rich, A. S. Boparai, and M. S. Bernatowitz, *Biochem. Biophys. Res. Commun.* **104**, 3535 (1982).
[23] D. W. Christianson and W. N. Lipscomb, *J. Am. Chem. Soc.* **108**, 4998 (1986).
[24] P. A. Bartlett and C. K. Marlowe, *Biochemistry* **26**, 8553 (1987).
[25] A. P. Kaplan and P. A. Bartlett, *Biochemistry* **30**, 8165 (1991).
[26] B. W. Matthews, *Acc. Chem. Res.* **21**, 333 (1988).
[27] M. R. Maurizi and A. Ginsburg, *J. Biol. Chem.* **257**, 4271 (1982).
[28] J. Crosby and G. E. Lienhard, *J. Am. Chem. Soc.* **92**, 5707 (1970).

starting materials, and it was suggested that much of the transition state stabilization by pyruvate dehydrogenase was due to the hydrophobic nature of the active site. Keto or thioketo substitution at C-2 of the thiamine ring resulted in compounds with sp^3 hybridization at C-2, causing the ring nitrogen atom to lose its positive charge. These compounds proved to be extremely effective inhibitors or pyruvate dehydrogenase,[29] as did reduced TPP.[30]

Similarly, methyl transfer reactions involving positively charged *S*-adenosylmethionine probably involve charge dispersal in the transition state. Several nonpolar inhibitors of polyamine biosynthesis, synthesized by alkylation of thioamines with 5′-deoxy-5′-chloroadenosine, are considered to owe their effectiveness to hydrophobicity.[31]

Multisubstrate Analogs

Many enzymes play the role of a marriage broker, binding two or more substrates in a spatial relationship that is conducive to reaction. Multisubstrate analogs are single molecules that imitate the binding determinants in such a complex but save the enzyme the trouble of gathering the substrates from dilute solution. The first multisubstrate analog inhibitor ever prepared appears to have been pyridoxylalanine, a strong inhibitor of pyridoxamine–pyruvate transaminase.[32] Other early examples include L-benzylsuccinate, an inhibitor of carboxypeptidase A[33]; Ap_5A, a strong inhibitor of adenylate kinase[34]; and an inhibitor of ether lipid biosynthesis that incorporates the elements of both an attacking and a leaving group.[35] The number of such inhibitors is now very extensive, and, as discussed above, some may represent chemically activated species. One such analog, phosphorylated methionine sulfoximine, an inhibitor of glutamine synthetase,[36] appears to be bound with a K_d value in the range of 10^{-20} M.[27]

Practical Uses of Transition State Analogs

Transition state and multisubstrate analog inhibitors include a number of enzyme antagonists of practical importance. From a medicinal stand-

[29] J. A. Gutowski and G. E. Lienhard, *J. Biol. Chem.* **251**, 2863 (1976).
[30] P. N. Lowe, F. J. Leeper, and R. N. Perham, *Biochemistry* **22**, 150 (1983).
[31] K. C. Tang, A. E. Pegg, and J. K. Coward, *Biochem. Biophys. Res. Commun.* **96**, 1371 (1980).
[32] W. B. Dempsey and E. E. Snell, *Biochemistry* **2**, 1414 (1963).
[33] L. D. Byers and R. Wolfenden, *J. Biol. Chem.* **247**, 606 (1972).
[34] G. E. Lienhard and I. I. Secemski, *J. Biol. Chem.* **248**, 1121 (1973).
[35] S. Hixson and R. Wolfenden, *Biochem. Biophys. Res. Commun.* **101**, 1064 (1981).
[36] W. B. Rowe, R. A. Ronzio, and A. Meister, *Biochemistry* **8**, 2674 (1969).

point, these inhibitors represent attractive targets for drug design because the transition state tends to be uniquely characteristic of one kind of enzyme reaction, whereas substrates are usually shared by two or more enzymes. Most successful of these, from a clinical standpoint, have been antihypertensive inhibitors of the angiotensin-converting enzyme, namely, captopril[37] and enalapril,[38] whose design was based on benzylsuccinate, an early multisubstrate analog inhibitor of carboxypeptidase A.[33] A second major use of transition state analogs is as haptens in the production of catalytic antibodies, discussion of which is beyond the scope of this chapter (for reviews, see Lerner and Benkovic[39] and Shokat *et al.*[40]). Finally, transition state analogs constitute promising ligands for affinity chromatography and, more interestingly, as eluants from conventional substrate affinity columns. By using progressively increasing low concentrations of a transition state analog, enzymes can be eluted according to the molecular turnover numbers.[41]

Characterizing Transition State Analog Complexes

The affinities of transition state analogs for enzymes can be measured by methods conventionally used to characterize simple reversible inhibitors; however, when binding is extremely strong, the concentration of inhibitor present in kinetic experiments is so low that correction must be made for mutual depletion of the free enzyme and free inhibitor,[42] and for the time required for enzyme–inhibitor complexes to come to equilibrium. In cases of very tight binding, the best approach is to measure the "on" rate by determining the second-order rate constant for the onset of inhibition and the "off" rate by measuring the first-order rate constant for release of radiolabeled inhibitor in the presence of a large excess of unlabeled inhibitor. The quotient can be used to determine K_d values in the femtomolar range, which are inaccessible by other methods. Details of this method are given in papers describing applications to determining

[37] D. W. Cushman, H. S. Cheung, E. F. Sabo, and M. A. Ondetti, *Biochemistry* **16**, 5484 (1977).
[38] A. A. Patchett, E. Harris, E. W. Tristam, M. J. Wyvratt, M. T. Wu, D. Taub, E. R. Peterson, T. J. Ikeler, and J. ten Broeke, *Nature (London)* **288**, 280 (1980).
[39] R. A. Lerner and S. J. Benkovic, *Chemtracts—Org. Chem.* **3**, 1 (1990).
[40] K. M. Shokat, M. K. Ko, T. S. Scanlan, L. Kochersperger, S. Yonkovich, S. Thraisivongs, and P. G. Schultz, *Angew. Chem., Int. Ed. Engl.* **29**, 1296 (1990).
[41] L. Andersson and R. Wolfenden, *J. Biol. Chem.* **255**, 11106 (1980).
[42] J. L. Webb, *in* "Enzyme and Metabolic Inhibitors" Vol. 1, p. 184. Academic Press, New York, 1963.

the affinity of biotin for avidin[43] and to characterizing inhibitors of ribulosebisphosphate carboxylase[44] and carboxypeptidase A.[25]

One implication of the theory described in Scheme 1 is that any alteration in structure of the substrate, or of the enzyme, which alters the value of k_{cat} or K_m should have a predictable effect on the binding affinity of an ideal transition state analog inhibitor that perfectly resembles $S^{\ddagger}$. If a change in the structure of the substrate does not affect the rate of the nonenzymatic reaction, then any effect on k_{cat}/K_m should be matched by a change in the affinity of the enzyme for an ideal transition state analog inhibitor. This experimental test of analogy has been passed by inhibitors of papain,[19] elastase,[20] and thermolysin.[45] Alterations in the structure of the enzyme have been examined in the same way. After mutations at the active site, carboxypeptidase A[46] and cytidine deaminase[47] show changes in affinities for transition state analog inhibitors that are closely related to changes in k_{cat}/K_m.

The energetic consequences of stabilizing interactions can be analyzed individually, at least in principle, by deleting one of the interacting groups from either the inhibitor or the enzyme, then examining the thermodynamic consequences of alteration for binding affinity. Kinetic constants such as k_{cat}/K_m are sometimes open to ambiguities of interpretation, because the position of the transition state may vary along the reaction coordinate as alterations are made in the structure of an enzyme or substrate. In contrast, binding affinities of competitive inhibitors offer the advantage of being true dissociation constants that lend themselves to rigorous interpretation. This relationship has been tested for several enzymes by varying the inhibitor[9] and in two cases by varying the structure of the enzyme by site-directed mutagenesis.[46,47]

Examination of enzyme–inhibitor complexes by NMR, revealing states of ionization of both partners in enzyme complexes with transition state analog inhibitors, can provide important indications of the presence of acid–base catalysis as described in the above discussion of analogs of anionic reaction intermediates. States of covalent hydration of inhibitors can be equally revealing, as described in the discussion below of nucleoside deaminases.

[43] N. M. Green, *Biochem. J.* **89**, 585 (1963).
[44] J. V. Schloss, *J. Biol. Chem.* **263**, 4145 (1988).
[45] P. A. Bartlett and C. K. Marlowe, *Biochemistry* **22**, 4618 (1983).
[46] M. A. Phillips, A. P. Kaplan, W. J. Rutter, and P. A. Bartlett, *Biochemistry* **31**, 959 (1992).
[47] A. A. Smith, D. Carlow, R. Wolfenden, and S. A. Short, *Biochemistry* **33**, 6468 (1994).

Slow Binding

Strongly bound inhibitors are often bound slowly: lactate oxidase, for example, binds oxalate with a rate of onset of 80 M^{-1} sec^{-1}.[48] Several caveats should be borne in mind in considering whether these properties are likely to bear any functional relationship to transition state analogy. First, when an inhibitor is bound with high affinity, slow binding (when that is also present) tends to be obvious, simply because the behavior of the inhibitor must be examined at very low concentrations to determine the value of K_i. If, for example, an inhibitor is bound with a K_i value of 10^{-10} M, and the rate constant for formation of EI is 10^7 sec^{-1} M^{-1}, then a slow rate of onset of inhibition becomes obvious in any kinetic investigation intended to determine K_i, carried out over a period of a few minutes. At a concentration of 10^{-10} M, the pseudo-first-order rate constant for the onset of inhibition would be 10^{-3} sec^{-1}. However, if another inhibitor combined with the enzyme at the same rate, but was much less strongly bound ($K_i = 10^{-5}$ M, for example), then its rate of onset of inhibition would be very difficult to determine. For this reason, it remains to be demonstrated in most cases whether "slow" binding is any less common among conventional substrate analog inhibitors than among transition state and multisubstrate analog inhibitors that are much more strongly bound. Second, some transition state analog inhibitors combine rapidly with enzymes, whereas others combine very slowly.[11] If slow binding were an essential, rather than an accidental, feature of transition state analogy, then such variation might not have been expected. Thus, the relationship between transition state affinity and slow binding, if such a relationship exists, is inconsistent, and its origins seem likely to be complex.

Several possible reasons might be advanced to explain slow binding. First, strongly bound inhibitors tend to contain several binding determinants, each of which must be properly engaged for optimal binding. These various interactions might take effect in stages, with some adjustment of the configuration of the active site or the inhibitor that requires the elapse of time. It is also possible that some binding determinants may engage more rapidly than others, resulting in formation of a weak "abortive" complex; this complex must first dissociate before it becomes possible to form the final complex that engages all the appropriate binding determinants. Although rapid weak binding is often followed by slow tight binding, it has seldom if ever been determined whether the rapid weak complex lies on the pathway to the slow tight complex.

[48] S. Ghisla and V. Massey, *J. Biol. Chem.* **250**, 577 (1975).

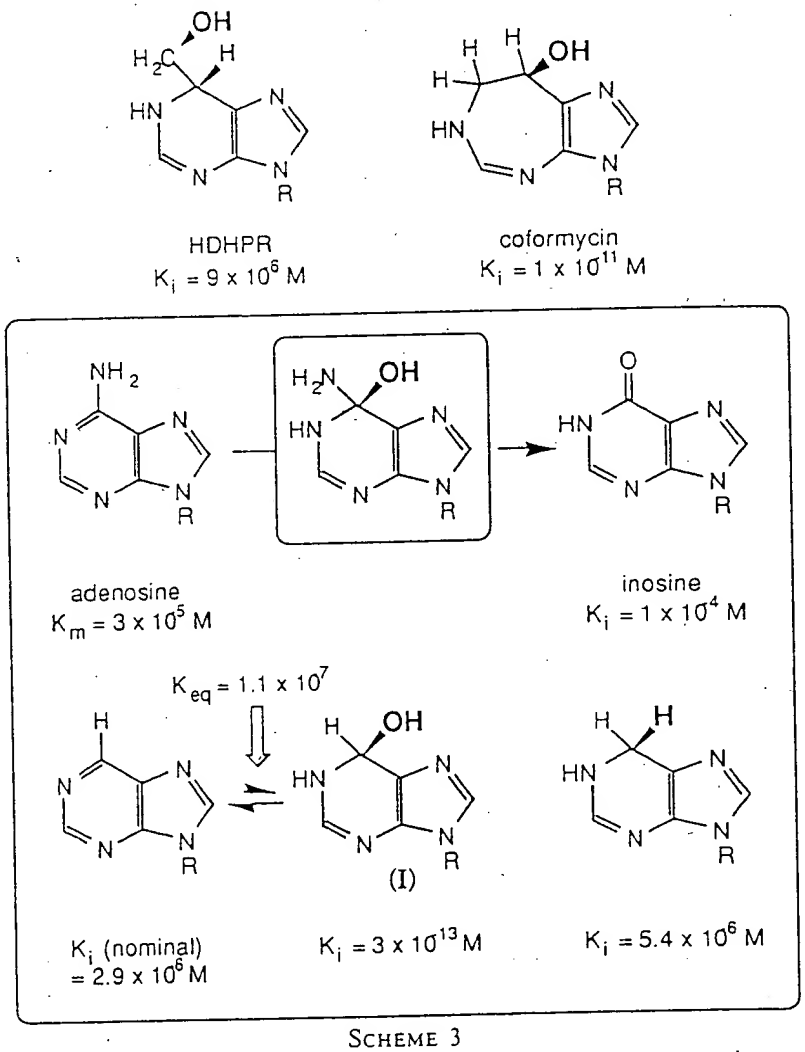
Another explanation for slow binding of transition state analogs may be that the enzyme has not been prepared by natural selection to bind, rapidly, a molecule that resembles the altered substrate in the transition state, although its affinity for that species is extremely high. Ordinarily, the transition state develops from the bound substrate or product, which the enzyme has been prepared by natural selection to bind rapidly, permitting it to act at rates that often approach the limits imposed by encounter between the substrate and enzyme in solution. Subsequent reactions, within the ES complex, may involve topologically complex transformations, including the closing of a lid or flap, to maximize the attractive interactions that so greatly stabilize the altered substrate in the transition state.[49] New crystal structures show that bound transition state analog inhibitors are enveloped so completely that they become almost completely inaccessible to solvent water in the cases of triose-phosphate isomerase,[14] adenosine deaminase,[50] and cytidine deaminase.[51]

Mechanistic Uses of Transition State Analogs: Two Case Histories

Hydrolytic deamination of adenosine, catalyzed by fungal and mammalian enzymes, is strongly inhibited by analogs of an unstable hydrated intermediate formed by 1,6-addition of substrate water approaching from the front side of the adenosine ring as viewed in Scheme 3. Thus, 1,6-hydroxymethyl-1,6-dihydropurine ribonucleoside (HDHPR) and the antibiotics coformycin and 2′-deoxycoformycin are powerful competitive inhibitors. Modeling studies show that the critical hydroxyl group of the hydroxymethyl substituent of the active isomer of HDHPR can be superimposed on the ring hydroxyl group of the natural 8*R*-OH isomer of 2-deoxycoformycin, both compounds being similar in structure to the postulated intermediate in the catalytic process.

In a remarkable display of steric discrimination, adenosine deaminase binds the natural 8*R*-OH isomer of 2-deoxycoformycin more tightly than the synthetic 8*S* isomer by a factor of 10^7.[52] This difference in affinity might arise from strong attraction of the 8*R* isomer by the active site, from steric hindrance of binding of the 8*S* isomer, or from some combination of these effects. In the 8*S* isomer, the critical hydroxyl group projects from the back side of the ring, from which the leaving group is believed to depart during the catalytic process. The extreme lack of specificity of the

[49] R. Wolfenden, *Mol. Cell. Biochem.* **3**, 207 (1974).
[50] D. K. Wilson, F. B. Rudolph, and F. A. Quiocho, *Science* **252**, 1278 (1991).
[51] L. Betts, S. Xiang, S. A. Short, R. Wolfenden, and C. W. Carter, Jr., *J. Mol. Biol.* **235**, 635 (1994).
[52] V. L. Schramm and D. C. Baker, *Biochemistry* **24**, 641 (1985).



SCHEME 3

enzyme with respect to the leaving group (NH_2-, CH_3NH_2-, Cl-, and CH_3O- are similarly reactive) suggests that the active site of the enzyme appears to be "as big as a barn" on the leaving group side, so that steric hindrance is improbable, and the first of these explanations seems most likely to be correct.

Purine ribonucleoside resembles the substrate adenosine except for replacement of the leaving -NH_2 group by hydrogen, and was long considered to be bound by adenosine deaminase as a simple competitive inhibitor with an affinity similar to the apparent affinity of the substrate. That view became untenable when ^{13}C NMR studies revealed that purine ribonucleoside was bound by adenosine deaminase with a change of hybridization

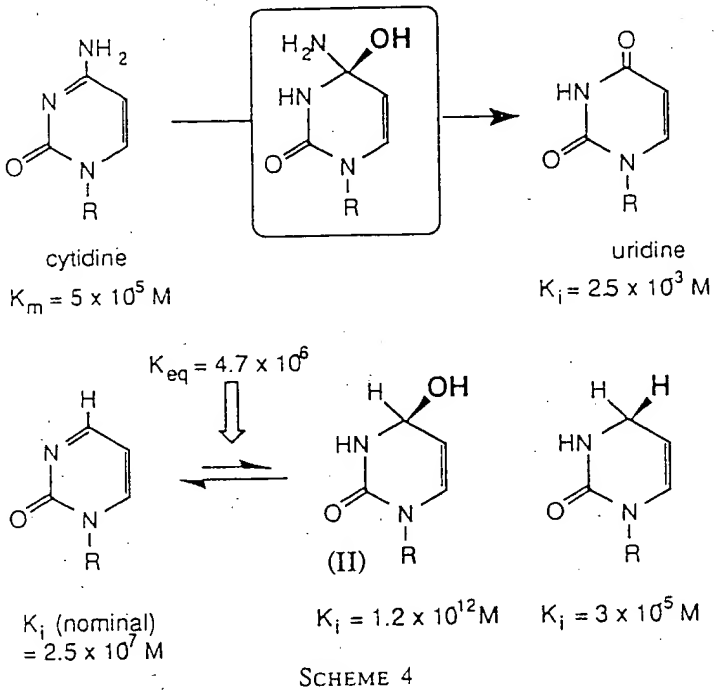
from sp^2 to sp^3 at C-6.[53] The NMR and UV spectra confirmed identification of enzyme-bound purine ribonucleoside as an oxygen adduct, presumably a 1,6-hydrate closely analogous in structure to the 1,6-hydrated intermediate in direct attack by water at the 6 position of adenosine.[5] In this structure, a hydrogen atom occupies the position presumed to be occupied by the leaving $-NH_2$ group in the normal reaction. Because the enzyme is nonspecific for leaving group (-Cl and $-NHCH_3$ are similarly reactive), it is also presumably indifferent to substitution by hydrogen at this position. If the apparent K_i value of purine ribonucleoside is combined with its extremely unfavorable equilibrium constant for hydration in free solution ($K_{eq} = 10^{-7}$), then the true K_i value of the more inhibitory of the two diastereomers of the 1,6-hydrate is found to be in the neighborhood of 3×10^{-13} M.[54]

From the rapid rate of onset of inhibition and the rarity of the hydrate in free solution, it was clear that inhibition normally occurs as a result of purine ribonucleoside binding, followed by hydration at the active site in a mockery of the normal catalytic process.[53] It could further be shown that the equilibrium of hydration is greatly enhanced at the active site of the enzyme, where the effective concentration of substrate water is approximately 10^{10} M.[9]

Cytidine deaminases from bacteria and mammals are strongly inhibited by 3,4,5,6-tetrahydrouridine, structurally analogous to a hypothetical intermediate formed by 3,4-addition of water to the alternate substrate 5,6-dihydrocytidine, shown in Scheme 4. The competitive inhibitors pyrimidin-2-one ribonucleoside [$K_{i(app)} = 3.6 \times 10^{-7}$ M] and 5-fluoropyrimidin-2-one ribonucleoside [$K_{i(app)} = 3.5 \times 10^{-8}$ M] exhibit UV absorption spectra, in their complexes with the enzyme, that are virtually identical with those of the products obtained when hydroxide ion combines with analogs quaternized at N-3.[6] These results indicate that the bound inhibitors are oxygen adducts and provide evidence in favor of binding as a covalent hydrate, not as an enzyme cysteine derivative that had been considered as an alternative possibility. The apparent K_i value of pyrimidin-2-one ribonucleoside as an inhibitor of bacterial cytidine deaminase, combined with its equilibrium constant for covalent hydration in free solution, indicates that $K_i = 1.2 \times 10^{-12}$ M for 3,4-dihydrouridine (the 3,4-hydrate of pyrimidin-2-one ribonucleoside).

Adenosine and cytidine deaminases are nonspecific for the leaving group in substrates, so that they are probably indifferent to replacement of the leaving group by hydrogen in analogs I and II and bind these

[53] L. Kurz and C. Frieden, *Biochemistry* **26**, 8450 (1987).
[54] W. Jones and R. Wolfenden, *J. Am. Chem. Soc.* **108**, 7444 (1986).



SCHEME 4

transition state analogs very tightly. Thus, the hydroxyl group at the sp^3-hybridized carbon atom probably offers one of the few structural features that could be used by either adenosine or cytidine deaminase to distinguish the altered substrate in the transition state for deamination from the substrate in the ground state (Schemes 3 and 4). To assess the contribution of this hydroxyl group to the binding of analogs I and II, we examined the results of replacement by hydrogen. 1,6-Dihydropurine ribonucleoside, prepared photochemically, was found to serve as a simple competitive inhibitor of adenosine deaminase, with a K_i value of 5.4×10^{-6} M. When this value was compared with the K_i value of the 1,6-hydrate of purine ribonucleoside (1.6×10^{-13} M), it became evident that the 6-hydroxyl group of the latter compound contributes -9.8 kcal/mol to the free energy of binding by calf intestinal adenosine deaminase (Scheme 3).[55,56]

Similar experiments on bacterial cytidine deaminase, performed with 3,4-dihydropyrimidin-2-one ribonucleoside ($K_i = 3.0 \times 10^{-5}$ M), showed that the 4-hydroxyl group of 3,4-dihydrouridine contributes -10.1 kcal/mol to the free energy of binding (Scheme 4).[6] Molecular orbital calculations suggest that the geometry and density of electrons are essentially identical at other positions in the hydrogen- and hydroxyl-substituted

[55] W. M. Kati and R. Wolfenden, *Science* **243**, 1591 (1989).
[56] W. M. Kati and R. Wolfenden, *Biochemistry* **28**, 7919 (1989).

ligands, so that these hydroxyl group contributions to binding affinity, approximately -10 kcal/mol, can be considered to result from simple replacement of -OH by -H.

Group Contributions and Role of Solvent Water

When bound by a protein, a ligand must normally be removed, at least in part, from solvent water. To compare the inherent affinities of the desolvated ligands for the active site, it would therefore be of interest to correct for the free energies of their prior removal from solvent water. (Binding also involves removal of the active site from previous contact with solvent water, but this is true in either case and does not contribute to the difference in affinities between the hydroxyl-containing and the hydrogen-containing ligands.) Free energies have now been determined for removal of many compounds of biological interest from solvent water, from their water-to-vapor or water-to-cyclohexane distribution coefficients. To a fair approximation, free energies of solvation of organic compounds are found to vary as an additive function of constituent groups, alcohols being solvated more strongly than the corresponding alkanes by a factor of roughly 10^5.[15] If a hydroxyl-containing ligand is more readily desolvated that the corresponding hydrogen-containing ligands by roughly 7 kcal/mol in free energy, then for both adenosine and cytidine deaminases the contribution of a desolvated hydroxyl group to the binding of a transition state analog inhibitor appears to be approximately -17 kcal/mol.

In arriving at this conclusion, we have assumed that solvent water has been stripped completely from ligands at critical points of contact with the enzyme. That assumption, although it seems plausible for the hydroxylated ligand whose high affinity implies a close fit to the active site, may not be appropriate in the case of the hydrogen-containing ligand. In the latter case a molecule of water may take the place of the missing hydroxyl group. This "trapping" of water would invalidate simple comparison of observed binding affinities as a measure of the contribution of the hydroxyl group to binding affinity. However, if water is trapped in this way, then the stability of the resulting "wet" complex of the hydrogen-containing ligand must presumably be greater than that of any hypothetical "dry" complex of the hydrogen-containing ligand, from which trapped water was absent. Otherwise, a "dry" complex, of the kind needed for direct comparison of binding affinities, would have been formed by the hydrogen-containing ligand. Under these circumstances, the observed difference in binding affinities would be less than the difference in "dry" binding affinities that is needed to determine the contribution of the hydroxyl group to ligand binding.

The meaning of our earlier estimate of the contribution of the critical hydroxyl group to binding, based on the difference in binding affinity between the two ligands, would also be clouded if the conformation of the enzyme were to change and, to a different extent, on binding of the different ligands. The strong affinity observed for the hydroxylated ligand suggests that the native conformation of the enzyme is already well adapted to tight binding of the hydroxyl-containing ligand. The hydrogen-containing ligand, being smaller, should be able to fit into any "native" structure that can accommodate the hydroxylated ligand. It would hardly be surprising, however, if the active site of the enzyme were to show some tendency to collapse around the hydrogen-containing ligand, forming a more compact structure than does the complex of the hydroxyl-containing ligand. Such a change in structure would invalidate simple comparison of binding affinities as a measure of hydroxyl group contribution to binding. If, however, the hydrogen-containing ligand were bound with such a change in conformation, then the stability of the resulting "collapsed" complex would necessarily be greater than that of any complex with the active site in the "native" configuration. Otherwise, the natively configured complex, being more stable, would have been the species actually observed at equilibrium. The contribution of the hydroxyl group to the stability of the complex of the hydroxyl-containing ligand in the native structure would again have been underestimated.

These considerations suggest that if "water trapping" or enzyme distortion accompany formation of the complex of the enzyme with the hydrogen-containing ligand, then either of these effects might be expected to exert a "leveling" influence on the relative affinities observed for the hydroxyl- and hydrogen-containing ligands, leading to underestimation of the contribution of the critical hydroxyl group to binding affinity. Evidently, the contributions of these hydroxyl groups to binding affinities of the desolvated ligands are probably at least as large, and could be larger, than values of approximately -17 kcal/mol suggested by the observed differences in K_i values.

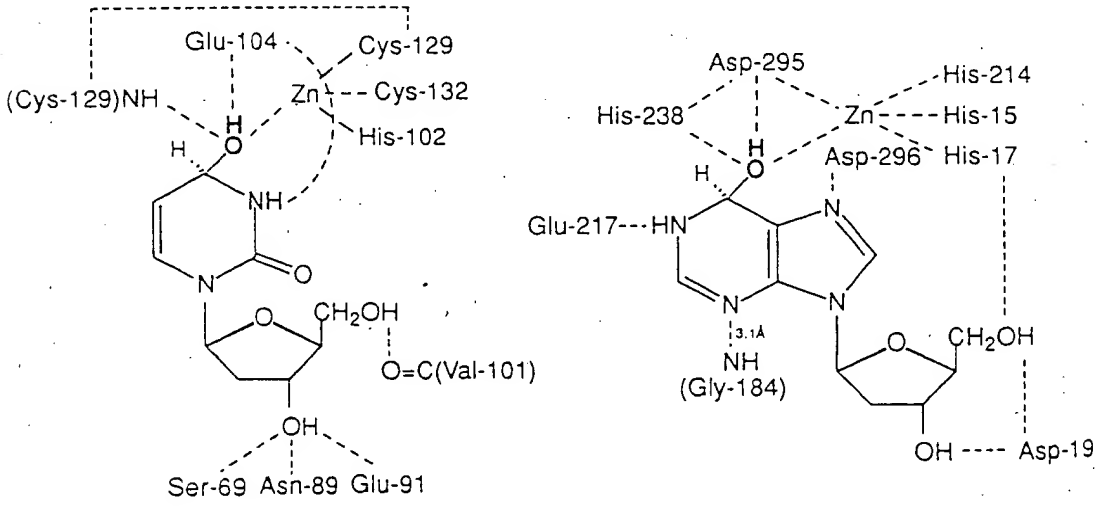
Crystal structures have now been reported for the complexes formed between transition state analogs and adenosine[57] and cytidine[51] deaminases. The results confirm that these inhibitors are bound as the covalent hydrate almost completely removed from contact with solvent water, as in the complex that is formed between 2-phosphoglycolate and triosephosphate isomerase.[14] The implied conformation change, by maximizing the possibility of attractive interactions between the enzyme and substrate in the transition state, may help to answer the conflicting requirements

[57] D. K. Wilson, F. B. Rudolph, and F. A. Quiocho, *Science* **252**, 1278 (1991).

of transition state stabilization and rapid access of substrates and egress of products.[49] Several features of the new crystal structures are shown in Scheme 5. The critical hydroxyl group of the inhibitor, on which so much of the catalytic binding enhancement appears to depend, interacts with three groups, including a zinc atom and a carboxylate residue at the active site of the enzyme.

Conclusions

In summary, many of the structural features of a substrate remain unchanged as it passes from the ground state to the transition state. To enhance the rate of a reaction, an enzyme must therefore single out for chemical recognition those few features of a substrate that do change. We have considered the generation of hydrates I and II at the active sites of nucleoside deaminases as analogs of the process by which such enzymes generate intermediates in substrate transformation. In these compounds, a tetrahedrally oriented hydroxyl group is an obvious feature that distinguishes these compounds from the aromatic starting materials. Evidently a few polar interactions involving this group, arising fleetingly in the transition state, are capable of generating a large part of the added binding affinity that is needed to explain the rate enhancement ($\sim 10^{12}$-fold) that an enzyme of this kind produces. Other interactions with the enzyme are obviously important in transition state stabilization and can be analyzed



bacterial cytidine deaminase *mammalian adenosine deaminase*

SCHEME 5

by similar methods, including active site directed and inhibitor directed mutagenesis. Particular mention should be made of the structural results, too extensive for present discussions, of crystallographic studies of other enzyme complexes with transition state and multisubstrate analog inhibitors, especially those of triose-phosphate isomerase,[14] thermolysin,[26] and carboxypeptidase A.[23]

Transition State and Multisubstrate Analogs (Table I)

The list of enzymes and inhibitors in Table I is organized according to EC classification, and an attempt has been made to cite the original reference in each case. A list of this kind is certain to contain errors of omission, for which the authors apologize.

TABLE I
TRANSITION STATE AND MULTISUBSTRATE ANALOGS

EC Number[a]	Enzyme[a]	Inhibitor[b]
1.1.1.1	Alcohol dehydrogenase	NAD^+ adduct[1]
1.1.1.27	L-Lactate dehydrogenase	NAD^+ adduct, oxalate,[2] oxalylethyl-NADH[3]
1.1.1.37	Malate dehydrogenase	NAD^+ adduct[1]
1.1.1.145	3β-Hydroxy-Δ^5-steroid dehydrogenase	4-Aza-4-methyl-5-pregnane-3,20-dione[4]
1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase	Threose-2,4-diphosphate[5]
1.2.7.1	Pyruvate synthase	Thiamine thiazolone and thiamine thiothiazolone pyrophosphates,[6] tetrahydrothiamine pyrophosphate,[7] acetylphosphonate[8]
1.4.1.1	Alanine dehydrogenase	Oxalylethyl-NADH[9]
1.4.1.2	Glutamate dehydrogenase	NAD^+ adduct[10]
1.5.1.3	Dihydrofolate reductase	Methotrexate[11]
1.5.1.X	Deoxyhypusine synthase	*N*-Guanyl-1,7-diaminoheptane[12]
1.11.1.9	Glutathione peroxidase	Mercaptosuccinate[13]
1.13.11.3	Protocatechuate 3,4-dioxygenase	2-Hydroxyisonicotinic acid *N*-oxide[14]
1.13.12.4	Lactate 2-monooxygenase	Oxalate,[15] malonate[16]
1.14.17.1	Dopamine β-monooxygenase	1-(4-Hydroxybenzyl)imidazole-2-thiol[17]
2.1.1.41	24-Sterol *C*-methyltransferase	25-Azacholesterol,[18] 24-(*R*,*S*)-epiminolanosterol,[19] 24-methyl-25-azacycloartanol[20]
2.1.1.45	Thymidylate synthase	1-(D-2′-Deoxyribofuranosyl)-8-azapurin-2-one 5′-monophosphate,[21] multisubstrate analog[22]
2.1.2.2	Phosphoribosylglycinamide formyltransferase	β-Thioglycinamide ribonucleotide dideazafolate[23]
2.1.3.1	Methylmalonyl-CoA carboxyltransferase	Oxalate[24]
2.1.3.2	Aspartate carbamoyltransferase	Phosphonoacetylaspartate[25]
2.1.3.3	Ornithine carbamoyltransferase	Phosphonoacetylornithine[26,27]
2.3.1.7	Carnitine *O*-acetyltransferase	*O*-[2-(*S*-Coenzyme A)acetyl]carnitine[28]
2.3.1.48	Histone acetyltransferase	*N*-[2-(*S*-Coenzyme A)acetyl]spermidine amide[29]
2.3.1.59	Gentamicin 2′-*N*-acetyltransferase	*N*-[2-(*S*-Coenzyme A)acetyl]gentamicin[30]
2.3.1.X	Succinyl-CoA: tetrahydrodipicolinate *N*-succinyltransferase	2-Hydroxytetrahydropyran-2,6-dicarboxylic acid[31]
2.3.2.2	γ-Glutamyltransferase	Serine–borate complex[32]
2.4.1.1	Glycogen phosphorylase	1,5-Gluconolactone,[33,34] 1-deoxy-α-D-*gluco*-heptulose 2-phosphate[35]
2.4.1.19	Cyclomaltodextrin glucanotransferase	Acarbose[36]
2.4.1.X	$\alpha(1 \rightarrow 2)$-Fucosyltransferase	2-*O*-(2-Guanosinophosphonoethyl)-1-*O*-(phenyl)-β-D-galactopyranoside[37]
2.4.2.1	Purine-nucleoside phosphorylase	8-Amino-2′-nordeoxyguanosine, 2′-nordeoxyguanosine diphosphate[38]
2.5.1.1	Geranylgeranyl diphosphate synthase	3-Azageranyl diphosphate[39]
2.5.1.6	Methionine adenosyltransferase	5′-(δ-Methylmethionine)-β,γ-imido-ATP[40]
2.5.1.9	Riboflavin synthase	6,7-Dioxolumazine[41]
2.5.1.16	Spermidine synthase	*S*-Adenosyl-3-thio-1,8-diaminooctane[42]
[illegible]	[illegible]	Carboxyallenyl phosphate, (*Z*)-3-fluorophosphoenolpyruvate[43]

2.3.2.2	γ-Glutamyltransferase	Serine–borate complex[32]
2.4.1.1	Glycogen phosphorylase	1,5-Gluconolactone,[33,34] 1-deoxy-α-D-*gluco*-heptulose 2-phosphate[35]
2.4.1.19	Cyclomaltodextrin glucanotransferase	Acarbose[36]
2.4.1.X	α(1 → 2)-Fucosyltransferase	2-*O*-(2-Guanosinophosphonoethyl)-1-*O*-(phenyl)-β-D-galactopyranoside[37]
2.4.2.1	Purine-nucleoside phosphorylase	8-Amino-2′-nordeoxyguanosine, 2′-nordeoxyguanosine diphosphate[38]
2.5.1.1	Geranylgeranyl diphosphate synthase	3-Azageranyl diphosphate[39]
2.5.1.6	Methionine adenosyltransferase	5′-(δ-Methylmethionine)-β,γ-imido-ATP[40]
2.5.1.9	Riboflavin synthase	6,7-Dioxolumazine[41]
2.5.1.16	Spermidine synthase	*S*-Adenosyl-3-thio-1,8-diaminooctane[42]
2.5.1.19	3-Phosphoshikimate 1-carboxyvinyltransferase	Carboxyallenyl phosphate, (*Z*)-3-fluorophosphoenolpyruvate[43]
2.5.1.21	Farnesyl-diphosphate farnesyltransferase	Ammonium analog of carbocation[44]
2.5.1.22	Spermine synthase	*S*-Adenosyl-1,12-diamino-3-thio-9-azadodecane[45]
2.5.1.26	Alkylglycerone-phosphate synthase	2(3)-Palmitoyl-1,2,3-trihydroxyeicosane 1-phosphate[46]
2.6.1.30	Pyridoxamine–pyruvate transaminase	Pyridoxylalanine[47]
2.7.1.1	Hexokinase	Chromium-ATP[48]
2.7.1.20	Adenosine kinase	Ap_4A[49]
2.7.1.21	Thymidine kinase	Ap_5T[48,50]
2.7.1.40	Pyruvate kinase	Oxalate[51]
2.7.3.2	Creatine kinase	Nitrate[52]
2.7.3.3	Arginine kinase	Nitrate[53,54]
2.7.4.3	Adenylate kinase	Ap_5A[55]
2.7.9.2	Pyruvate, water dikinase	Oxalate[56]
2.7.10.X	p70 S6 kinase	Rapamycin[57]
2.8.3.5	3-Oxoacid CoA-transferase	Succinic monohydroxamic acid[58]
3.1.1.1	Carboxylesterase	Benzil,[59] ethylphenyl glyoxalate[60]
3.1.1.4	Phospholipase A_2	1-Hexadecylthio-2-hexadecanoylamino-1,2-dideoxy-*sn*-glycero-3-phosphocholine, 1-hexadecylthio-1-deoxy-2-hexadecylphosphono-*sn*-glycero-3-phosphocholine,[61] 1-hexadecyl-3-trifluoroethylglycero-*sn*-2-phosphomethanol[62]
3.1.1.7	Acetylcholinesterase	Boronate,[63] fluoroketone[64,65]
3.1.1.8	Cholinesterase	Diphenylboric acid[66]
3.1.1.13	Sterol esterase	Phenyl-*n*-butylborinic acid,[67] chlorodifluoroacetophenone[68]
3.1.1.34	Lipoprotein lipase	Phenyl-*n*-butylborinic acid[69]
3.1.1.59	Juvenile-hormone esterase	Fluoroketones[70]
3.1.3.1	Alkaline phosphatase	Vanadate[71]
3.1.3.2	Acid phosphatase	Tungstate, molybdate[72]
3.1.6.1	Arylsulfatase	Sulfite[73]

(*continued*)

TABLE I (*continued*)

EC Number[a]	Enzyme[a]	Inhibitor[b]
3.1.27.5	Pancreatic ribonuclease	Uridine-vanadate[74]
3.2.1.1	α-Amylase	D-*malto*-Bionolactone[75]
3.2.1.10	*O*-Glycosyl glycosidase	Acarbose,[76] bis(hydroxymethyl)dihydroxypyrrolidine[77]
3.2.1.17	Lysozyme	Lactone, 2-acetamido-2-deoxyglucoseen[78]
3.2.1.18	Exo-α-sialidase	2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid[79]
3.2.1.21	β-Glucosidase	1-Aminoglucoside,[80] gluconolactone[81]
3.2.1.22	Galactosidase	1-Aminogalactoside,[82] galactal[83]
3.2.1.23	β-Galactosidase	1-Aminogalactoside,[82] galactal[83]
3.2.1.45	Glucosylceramidase	Glucosylamine[84]
3.2.1.48	Sucrose α-glucosidase	Castanospermine,[85] 2,6-Dideoxy-2,6-imino-7-*O*-(β-D-glucopyranosyl)-D-*glycero*-L-*gulo*-heptitol.[86]
3.2.1.52	β-*N*-Acetylhexosaminidase	NAG-lactone[87]
3.2.1.55	α-*N*-Arabinofuranosidase	L-Arabino-1,4-lactone[88]
3.2.2.4	AMP nucleosidase	Formycin 5′-phosphate[89]
3.2.2.X	Uracil-DNA-glycosylase	6-(*p*-*n*-Octylanilino)uracil[90]
3.4.11.1	Leucyl aminopeptidase	Aminoaldehydes,[91] bestatin,[92] amastatin,[93] aminohydroxamates[94]
3.4.15.1	Peptidyl-dipeptidase A	Captopril,[95] enalapril,[96] fluoroketone,[97] aminoalcohol,[98] 2-mercaptoacetyl dipeptides,[99] ketodimethyl peptide,[100] aminoketodimethyl peptides[101]
3.4.17.1	Carboxypeptidase A	Benzylsuccinate,[102] L-2-phosphoryloxy-3-phenylpropionic acid,[103] dipeptide phosphoramidates,[104] 2-benzyl-3-formylpropionate, 2-mercaptoacetyl dipeptides,[76] 3-phosphonopropionic acid,[105] (2-carboxy-3-phenylpropyl)methylsulfoximine and -sulfodiimine,[106] phosphonotetrapeptides[107]
3.4.17.2	Carboxypeptidase B	Benzylsuccinate[108]
3.4.17.3	Lysine carboxypeptidase	2-Mercaptomethyl-3-guanidinoethyl thiopropionate,[109] phosphono dipeptide[110]
3.4.19.3	Pyroglutamyl-peptidase I	Oxoprolinal[111]
3.4.21.1	Chymotrypsin	Aldehyde,[112] 1-acetamido-2-phenylethaneboronic acid,[113] peptidyl fluoromethyl ketones[114]
3.4.21.4	Trypsin	Peptide and nonpeptide borates[115]
3.4.21.36	Pancreatic elastase	Peptidyl fluoromethylketones,[116] acetyl-Pro-Ala-Pro-alaninal[117]
3.4.21.62	Subtilisin	Benzeneboronic acid[118]
3.4.22.2	Papain	Acetyl-Phe-glycinal,[119] *N*-acetyl-1-phenylalanylaminoacetonitrile[120]
3.4.22.3	Ficain	Acetyl-Phe-glycinal[121]
3.4.23.1	Pepsin A	Pepstatin,[122] methylpepstatin and statone,[123] fluoroketone,[124] phosphinic acid dipeptide[125]

	Chymotrypsin	Aldehyde,[112] 1-acetamido-2-phenylethaneboronic acid,[113] peptidyl fluoromethyl ketones[114]
3.4.21.4	Trypsin	Peptide and nonpeptide borates[115]
3.4.21.36	**Pancreatic elastase**	**Peptidyl fluoromethylketones,[116] acetyl-Pro-Ala-Pro-alaninal[117]**
3.4.21.62	**Subtilisin**	**Benzeneboronic acid[118]**
3.4.22.2	**Papain**	**Acetyl-Phe-glycinal,[119] *N*-acetyl-1-phenylalanylaminoacetonitrile[120]**
3.4.22.3	**Ficain**	**Acetyl-Phe-glycinal[121]**
3.4.23.1	Pepsin A	Pepstatin,[122] methylpepstatin and statone,[123] fluoroketone,[124] phosphinic acid dipeptide[125]
3.4.23.15	Renin	Statine,[126] difluorostatine and difluorostatone,[127] reduced peptide,[128] dihydroxyethylene peptide analogs[129]
3.4.23.X	HIV protease	Hydroxyethylene peptide isostere[130]
3.4.24.3	Microbial collagenase	Isoamylphosphonyl peptide,[131] cinnamoyl-Phe-(CO)Gly-Pro-Pro ketone,[132] phosphonamide[133]
3.4.24.11	Neprilysin	*N*-Carboxymethyl peptides,[137] 2-mercaptoacetyl dipeptides[76]
3.4.24.27	Thermolysin	*N*-Carboxymethyl dipeptides,[134] phosphonamidate,[135] dipeptides,[76] hydroxamates[83,136]
3.5.1.1	Asparaginase	Aspartate semialdehyde[138]
3.5.1.4	Amidase	Acetaldehyde–ammonia[139]
3.5.2.3	Dihydroorotase	Borocarbamylethyl aspartate[140]
3.5.2.6	β-Lactamase	Arylmethyl phosphonate methyl ester[141]
3.5.4.3	Guanine deaminase	(1,2,6)-Thiadiazine-1,1-dioxides[142]
3.5.4.4	Adenosine deaminase	1,6-Dihydro-6-hydroxymethylpurine ribonucleoside,[143] coformycin,[144] 1,6-dihydroinosine[145]
3.5.4.5	Cytidine deaminase	Tetrahydrouridine,[146] phosphapyrimidine,[147] 1,3-diazepin-2-ol ribonucleoside,[148] 3,4-dihydrouridine[148a]
3.5.4.6	AMP deaminase	Coformycin 5′-phosphate[149]
3.5.4.12	dCMP deaminase	Tetrahydrouridine 5′-phosphate[150]
3.11.1.1	Phosphonoacetaldehyde hydrolase	Phosphite + acetaldehyde[151]
	Cholesterol 5,6-oxide hydrolase	5,6-Iminocholestanol[152]
4.1.1.3	Oxaloacetate decarboxylase	Oxalate[153]
4.1.1.4	Acetoacetate decarboxylase	Acetopyruvate,[154] acetoacetone[155]
4.1.1.23	Orotidine-5′-phosphate decarboxylase	1-(5′-Phospho-D-ribosyl)barbituric acid[156]
4.1.1.39	Ribulose-bisphosphate carboxylase	Carboxyribitol diphosphate,[157] carboxyarabinitol diphosphate[158]
4.1.2.13	Fructose-bisphosphate aldolase	Phosphoglycolohydroxamate[159]
4.1.3.1	Isocitrate lyase	3-nitropropionate[160]
4.1.3.7	Citrate (*si*)-synthase	Carboxymethyl-CoA and oxaloacetate,[161] *S*-acetonyl-CoA[162]
4.1.99.1	Tryptophanase	2,3-Dihydrotryptophan[159]
4.1.99.4	1-Aminocyclopropane-1-carboxylate deaminase	1-Aminocyclopropane phosphonate[163]

(*continued*)

TABLE I (*continued*)

EC Number[a]	Enzyme[a]	Inhibitor[b]
4.2.1.2	Fumarate hydratase	3-Nitro-2-hydroxypropionate,[161] *S*-2,3-dicarboxyaziridine[165]
4.2.1.3	Aconitate hydratase	Nitro analogs of citrate and isocitrate[166]
4.2.1.17	Enoyl-CoA hydratase	Acetoacetyl-CoA[167]
4.2.1.20	Tryptophan synthase	2,3-Dihydrotryptophan[168]
4.3.1.1	Aspartate ammonia-lyase	3-Nitro-2-aminopropionate[169]
4.3.1.5	Phenylalanine ammonia-lyase	L-2-Aminooxy-3-phenylpropionic acid[170]
4.3.2.2	Adenylosuccinate lyase	N^6-(L-2-Carboxyethyl-2-nitroethyl)-AMP[171]
4.4.1.5	Lactoylglutathione lyase	3-Hydroxy-2-methyl-4*H*-pyran-4-one, isoascorbate,[172] isopropyltropolon[173]
5.1.1.1	Alanine racemase	1-Aminocyclopropane phosphonate[164]
5.1.1.4	Proline racemase	Pyrrole,[174] pyrroline 2-carboxylates[175]
5.1.1.7	Diaminopimelate epimerase	3-Chlorodiaminopimelic acid[176]
5.3.1.1	Triose-phosphate isomerase	2-Phosphoglycolate,[177] 2-phosphoglycolohydroxamate[178]
5.3.1.6	Ribose-5-phosphate isomerase	4-Phosphoerythronate[179]
5.3.1.9	Glucose-6-phosphate isomerase	5-Phosphoarabinonate[180]
5.3.1.13	Arabinose-5-phosphate isomerase	4-Phosphoerythronate[181]
5.3.3.1	Steroid Δ-isomerase	17β-D-hydroequinalin[182]
5.3.3.2	Isopentenyl-diphosphate Δ-isomerase	2-(Dimethylamino)ethyl pyrophosphate[183]
5.4.2.2	Phosphoglucomutase	α-D-glucose 1-phosphate vanadate[184]
5.4.99.5	Chorismate mutase	Oxabicyclo[3.3.1]nonene,[185] 2-aza-2,3-dihydrosqualene[186]
6.3.1	Aminoacyl-tRNA ligases	Aminoalkyl adenylates,[187] aminophosphonyl adenylates[188]
6.3.1.2	Glutamate–ammonia ligase	Phosphinotricin,[189] methionine sulfoximine phosphate[190]
6.3.2.2	Glutamate–cysteine ligase	Buthionine-sulfoximine phosphate[191]
6.4.1.1	Pyruvate carboxylase	Oxalate[192]

[a] Classification and nomenclature of enzymes based on recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, published in 1992 (E. C. Webb, ed., Academic Press).

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(*continued*)

TABLE I (*continued*)

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